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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Intracellular Delivery of Biochemical Agents

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Notice: This application is as filed and may therefore contain an incomplete specification.

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ABSTRACT

The intracellular delivery of biochemical agents, such as therapeutic peptides and oligonucleotides, is facilitated by a carrier peptide coupled therewith. The carrier peptide consists desirably of positively charged D-amino acids. In a preferred embodiment, the carrier peptide consists of 8 or 9 D-arginine residues.

INTRACELLULAR DELIVERY OF BIOCHEMICAL AGENTS

Field of the Invention

This invention relates to carrier peptides useful to deliver to an intracellular location various biochemical agents, such as protein-based and nucleic acid-based drugs and diagnostic agents. More particularly, the invention relates to chemical conjugates, in which a selected agent is coupled chemically to a carrier peptide which facilitates delivery of the agent to an intracellular location, especially the cell nucleus.

Background to the Invention

The types of pharmaceutical agents available for diagnosing, treating and curing disease now varies widely, with the advent and successful application of various new biotechnological techniques. In addition to the traditional "small molecules" prepared by organic chemical synthesis, the pharmaceutical and diagnostic industries now have at their disposal such agents as recombinant protein products as well as rationally designed peptide compounds that are able to modulate selected genetic events Similarly, nucleic acid-based compounds have been rationally designed for use either as therapeutics or as diagnostic agents, to modulate specific cellular events or to probe for genetic information of diagnostic value.

Unlike the traditional pharmaceutical compounds however, many of the more recently designed agents are only poorly taken up by the intended target cells and tissues. As a result, therapeutic efficacy of many current drugs is inadequate, and administration of large and potentially toxic drug doses are required in order to elicit clinically beneficial effects.

It would accordingly be desirable to provide means for facilitating the uptake of biochemical agents targeted for intracellular delivery.

Summary of the Invention

In the present invention, a biochemical agent selected for intracellular delivery is coupled chemically to a peptide carrier which facilitates cellular uptake of the selected agent. Particularly, the present invention employs as carrier a peptide comprised mainly of positively charged D-amino acids. It has been found that such peptides are particularly amenable to cellular uptake, and are capable additionally of localizing in the cell nucleus.

More particularly, and according to one aspect of the present invention, there is provided a chemical conjugate comprising a biochemical agent selected for delivery to an intracellular location, and especially an intranuclear location, and a carrier peptide coupled chemically therewith to facilitate cellular uptake of the selected biochemical agent, wherein the carrier peptide comprised principally of positively charged amino acids, at least 50% of which are in the D-amino acid form.

Such carrier peptides can be utilized to facilitate cellular uptake and nuclear delivery of various biochemical agents, including protein-based and nucleic acid-based drugs and diagnostic agents. According to one embodiment of the present invention, the biochemical agent selected for delivery is one capable of regulating gene expression. Such biochemical agents include, for example, polynucleotides which hybridize to a region of genomic DNA which participates in expression of an undesirable gene prod so-called "anti-sense polynucleotides"), and polypeptides which interfere with protein/nucleic acid interactions that mediate expression of undesirable gene products, such as gene products of viruses and other pathogens, as well as products of aberrant human and other mammalian genes. The strategy herein described may also be applied to introduce such other biochemical agents as (poly)saccharides, as well as small molecule pharmaceuticals such as steroids and NSAIDS, and diagnostic agents such as labelled molecules.

In another aspect of the present invention, there is provided a method effective to introduce a biochemical agent into the nucleus of mammalian cells, which comprises the step

or bringing into contact with the cells a chemical conjugate comprising the biochemical agent and a carrier peptide coupled chemically therewith, wherein the carrier peptide is comprised principally of positively charged amino acids, at least 50% of which are D-amino acids.

In a further aspect of the present invention, a pharmaceutical composition is provided including a pharmaceutically acceptable excipient compound in combination with a chemical conjugate comprising a biochemical agent selected for delivery to an intracellular location and a carrier peptide coupled chemically therewith to facilitate cellular uptake of the selected biochemical agent, wherein said carrier peptide is comprised principally of positively charged amino acids, at least 50% of which are in the D-isomer form.

These and other aspects of the present invention are now described with reference to the accompanying drawings, in which:

Brief Reference to the Drawings

Figures 1 and 2 illustrate tissue distribution of a carrier peptide of the present invention.

Detailed Description of the Invention and Preferred Embodiments

The invention relates to chemical conjugates in which a carrier peptide amenable to cell uptake is coupled chemically with a biochemical agent selected for intracellular delivery.

To achieve intracellular delivery of the selected biochemical agent, the present invention employs as carrier a peptide that is comprised principally of independently selected positively charged amino acids, at least 50% of which are in the non-naturally occurring, D-form. The term "positively charged" amino acid refers to an amino acid having a side chain that is cationic in nature at neutral pH and in aqueous solution. Such positively charged amino acids include, for example, the naturally occurring amino acids such as arginine and lysine, as well as analogues thereof which retain a positively charged side chain.

With respect to carrier peptide composition, the term "comprised principally of" positively charged amino acids refers to a carrier peptide in which at least 2/3, more desirably 9/10 of the amino acids resident in the carrier peptide are positively charged amino acids. That is, it should be understood that the ability of the carrier peptide to facilitate uptake can be retained despite the incorporation of negatively charged or neutral charged amino acids. The limit of non-positively charged amino acid incorporation will of course depend on the relative number of positively charged amino acids in the carrier peptide, and thus on the length of the carrier peptide. The number of amino acids in the carrier peptide is chosen so that efficiency of cellular uptake of the conjugate is optimized. Generally, the carrier peptide will comprise at least three amino acids, up to about 30 amino acids. A greater number of amino acids may be incorporated, although efficiencies of production may be reduced.

According to one embodiment of the invention, the carrier peptide consists of from 5 to 10 independently selected, positively charged amino acids. In a preferred embodiment, the carrier peptide consists of 7, 8 or 9 independently selected, positively charged amino acid residues. According to a specific embodiment of the invention, amino acid components of the carrier peptide are selected independently from lysine or arginine. In a preferred embodiment of the invention, the carrier peptide consists of arginine residues.

Of the amino acid components of the carrier peptide, at least 50% are in the D-form. That is, a random mixture of L-form and D-form amino acids may be incorporated. Desirably, a greater proportion of the amino acids are in the stability-enhanced D-form. According to a preferred embodiment of the invention, the carrier peptide consists essentially of positively charged D-form amino acids.

According to a particularly preferred embodiment of the present invention, the carrier peptide consists of 7, 8 or 9 arginines, all of which are in the D-form.

To exploit the facility with which the carrier peptide is taken up by cells, the selected biochemical agent is coupled either directly or through a linking agent to one of the flanks of the carrier peptide, ie. either to the C-terminus or to the N-terminus, or is linked using a cross-linking agent to one of the derivatizable functional groups available on the carrier peptide, e.g., a functional group on an amino acid side chain.

It will be appreciated by one of skill in the art that the chemical conjugate of the present invention may include multiple biochemical agents per carrier peptide. This is particularly feasible in those instances where several similar reactive sites exist on a carrier peptide. For example, a poly-arginine carrier peptide includes not only an amino group at its N-terminus, but also includes multiple amino groups in the R-groups of arginine which all have the potential to react with a selected biochemical agent.

A variety of biochemical agents may be coupled to the carrier peptide to facilitate their delivery to the intracellular environment. For example, the biochemical agent selected for delivery may be one capable of modulating gene expression, usually in a way that is advantageous for disease treatment. A variety of gene regulating biochemical agents are now under clinical and research investigation, including oligonucleotides and polypeptides.

Oligonucleotides selected for intracellular delivery may be modulators e.g. antagonists, of gene expression which hybridize with regions of genomic DNA that mediate expression, for example of undesirable gene products. The so-called anti-sense oligonucleotides represent one such class of oligonucleotides that may be selected for intranuclear delivery using a carrier peptide of the present invention. These anti-sense oligonucleotides hybridize to regions for example of proviruses, and frustrate the transcriptional event leading to viral replication Alternatively, the oligonucleotides may be useful as genetic probes which hybridize to a gene or gene region that is diagnostic of an aberrant genetic composition. For diagnostic oligonucleotides, a reporter molecule will typically be incorporated on the oligonucleotide, so that its location can be detected following binding to the probed site.

The carrier peptide may also be coupled to a selected peptide agent, to facilitate cellular uptake of the peptide agent. A wide variety of peptide agents may be employed, including those having therapeutic utility. According to one embodiment of the invention, the peptide agent is an anti-viral peptide. Among the anti-viral peptides that may be delivered using the carrier peptide strategy herein described are domains of the HIV proteins such as gp120 and gp160, and anti-herpetic peptides such as the sequence Val-Val-Asn-Asp-Leu (see US 4,845,195).

Other peptides that may usefully be delivered intracellularly through the aid of a conjugated carrier peptide include those useful as vaccines, such as a peptidic antigen from a microbial pathogen e.g. virus or bacterium, that when delivered intracellularly raises an immune response following elaboration of the antigen on the target cell surface.

For coupling to the selected biochemical agent, the carrier peptide may first be prepared using the established solid- or solution phase techniques of protein synthesis now standard in the art, and recited in our co-pending published PCT patent application WO 92/07871. Once obtained, the carrier peptide may be released and isolated for subsequent coupling to a biochemical agent selected for delivery. Alternatively, in the case where the selected biochemical agent is itself a peptide, the chemical conjugate can be synthesized *in toto* using the peptide synthesis technique. In this case, the peptidic agent may occupy either the C-terminus or the N-terminus of the chemical conjugate.

Where the selected biochemical agent is non-peptidic, e.g. an oligonucleotide, each of the carrier peptide and oligonucleotide are synthesized separately and subsequently combined for chemical coupling. The synthesis of oligonucleotides, either of RNA or DNA structure, by automated production is now well established in the art and various strategies are available. For relatively long oligonucleotides, the block ligation approach may be employed, whereby "blocks" of oligonucleotide pairs are synthesized and ligated in correct succession by overhang complementarity. Alternatively, an oligonucleotide of the desired

sequence may be synthesized directly and then amplified, in intact form, using the polymerase chain reaction and oligonucleotide primers that anneal to the template flanks.

To produce the desired chemical conjugate, in which the carrier peptide is coupled functionally to the oligonucleotide to facilitate its intracellular delivery, procedures established in the art for the coupling of peptide to nucleic acid may be employed. This generally entails the linking, via a bifunctional reagent, of the C-terminal carboxyl group of the carrier peptide to the 5' hydroxyl group of the chosen oligonucleotide. Diols are particularly useful for this purpose, including ether and alkyl diols comprising from 2 to about 30 alkyl groups in the chain. A procedure for coupling a peptide to an oligonucleotide is described in WO89/02932 published April 6, 1989, and may also be employed, *mutatis mutandis*, for the purpose of coupling the carrier peptide to the selected oligonucleotide.

The coupling of biochemical agents, other than peptides and oligonucleotides, to carrier peptides to form chemical conjugates in accordance with the present invention are also encompassed. An example of such a biochemical agent is the drug methotrexate which may be condensed with the peptide carrier to form a methotrexate-carrier conjugate. Briefly, the condensation reaction entails combining appropriate amounts of methotrexate, N-hydroxysuccinimide and a carrier peptide, e.g. a polyarginine peptide, in a 10% pyridine/DMSO solvent. Once the solids are dissolved, a suitable amount of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide is added to the reaction mixture, the mixture is incubated for up to 30 minutes and following incubation, 5 volumes of water are added. The conjugate may then be isolated from any unreacted compounds using methods well-known in the art, for example, reverse-phase liquid chromatography. The methotrexate-carrier conjugate may include multiple methotrexate molecules per carrier peptide if the carrier includes several reactive amino groups because the reaction involves condensing the glutamate residue of methotrexate with an available reactive amino group on the carrier peptide.

Localization studies, as herein described have revealed significant liver accumulation of the peptide. Accordingly, it is expected that the carrier will be particularly useful for

delivering to hepatic tissue such therapeutic agents as are useful in treating cirrhosis and in combatting viral infection such as hepatitis.

In another aspect of the present invention, a pharmaceutical composition containing the chemical conjugate, consisting of the carrier peptide coupled to a chosen biochemical agent, is provided. Such compositions can be in any form suitable for administration, including tablets, pills, capsules, powders, aerosols, suppositories, creams, lotions, ointments, skin patches, parenterals, oral liquids such as suspensions, solutions and emulsions, and injectable liquids. The preferred administrable form of the composition will vary with the condition to be treated.

The composition additionally comprises a pharmaceutically acceptable excipient. As used herein, the term "pharmaceutically acceptable" means acceptable for use in the pharmaceutical and veterinary arts, and not being toxic or otherwise unacceptable. The nature of the excipient will vary with the intended mode of administration which will also vary with the condition to be treated. Thus, compositions to be administered orally are prepared using excipients that are suitably combined with the conjugate for oral ingestion, including but not limited to sugars, starches, cellulose and derivatives thereof, wetting agents, lubricants such as sodium lauryl sulfate, stabilizers, tabletting agents, anti-oxidants, preservatives, colouring agents and flavouring agents; while compositions to be administered by injection are prepared by combination with liquid excipients including, for example, buffered or physiological saline solutions.

The present pharmaceutical composition comprises a therapeutically effective amount of the chemical conjugate. The term "therapeutically effective amount" is used herein to denote an amount of chemical conjugate which includes respectively an amount of the active biochemical agent indicated for a given treatment and an amount of carrier peptide, the amounts of each not exceeding an amount which may cause significant adverse effects.

Specific embodiments of the present invention are described in the following Examples which are not to be construed as limiting.

Example 1 - Synthesis of acetyl-[D-Arg]$_9$-NH_2

The title compound, designated compound 4C, was synthesized using p-methylbenzhydrylamine (MBHA) resin as solid support, to provide the C-terminal blocking amine on the resultant peptide. Synthesis proceeded using D-arginine residues in which the amino function was blocked with the t-BOC group, and the guanidino function was blocked with the Tos group. Couplings were carried out using excess hydroxybenzotriazole (HOBt)-activated ester of BOC-L-Arg(Tos). Removal of the BOC protecting group after each cycle was effected with TFA. When coupling cycles were completed, the resin-bound peptide was treated with 20% acetic anhydride in acetonitrile, to incorporate an acetyl protecting group at the N-terminus thereof. Liberation of peptide from the resin, and removal of Tos groups, were achieved by treatment with hydrofluoric acid, yielding the C-terminally amidated, title compound. After removal of hydrofluoric acid, the resin/peptide mixture was washed with diethyl ether and extracted with aqueous acetic acid. The crude peptide was lyophilized, then fractionated by RP-HPLC on a C_{18} silica column using a gradient of 2-40% acetonitrile in 0.1% TFA. Fractions were collected and checked by analytical RP-HPLC. Those containing $\geq$95% of the major product were combined. High resolution mass spectrometry showed the product to the desired compound.

In the manner substantially as just described, but continuing synthesis cycles as appropriate, there is generated a chemical conjugate of the sequence acetyl-[L(Val-Val-Asn-Asp-Leu)$_1$-(D-Arg)$_9$-NH_2, which is useful for the treatment of herpesvirus.

Example 2 - Evidence for uptake of carrier peptide

Carbon 14 labelled peptide, produced as described in example 1, was added to cell culture growth medium (10% fetal bovine serum (FBS), 10 ug/ml gentamicin, 90% Dulbecco's modified MEM(DMEM for adherent cells and 10% FBS, 10 ug/ml gentamicin 90% Iscove's medium (IMDM for human T-cell lines)) at a concentration of 10 uM. The specific activity of the labelled peptide was 2,926 CPM/nmole. These media were added to flasks of growing monolayers of HeLa cells and Hut 78 cells (human T-cell line) respectively. Duplicate flasks of each cell line were processed at specified times after addition of medium containing labelled peptide (a total of 8 nmoles was added) and the amount of peptide taken up by the cells determined. The amount of labelled carrier peptide taken up by the cells was then determined as follows:

1. The medium was removed and the cells were washed twice by centrifugation with phosphate buffered saline (PBS). Adherent cells were removed from the surface of culture flasks using PBS containing 2 mM EDTA before the PBS washes.

2. The washed cell pellets were resuspended in 1 ml of 10 mM Tris-HCl pH 7.5, 3 mM MgCl2, 10 mM NaCl, 0.5% NP-40. These cell suspensions were placed on ice for 15 minute during which the outer membrane of the cells burst and releases the cytoplasmic contents of the cell leaving the nuclei intact.

3. The nuclei were pelleted by centrifugation at 2000 x g for 5 minutes.

4. The cytoplasm and nuclei were separated and placed in scintillation vials containing 10 ml of the scintillation cocktail Ecolume, and the amount of carrier peptide in the cytoplasm and nuclei of the cells was then analyzed.

Results of the experiment, which clearly demonstrate intracellular accumulation of the peptide, and significant nuclear accumulation, are tabulated below:

HeLa cell line

FRACTION	4 HOURS	24 HOURS	48 HOURS	1 WEEK
medium	12798	9884	9490	10116
wash	1198	907	943	1813
cytoplasm	635	2801	2641	2585
nuclei	293	926	1002	1353
cell total	928	3727	3643	3938
% uptake	6.22	25.67	25.88	24.82

Hut 78 cell line

FRACTION	4 HOURS	24 HOURS	48 HOURS	1 WEEK
medium	15575	14121	12705	10191
wash	651	584	827	1088
cytoplasm	772	1061	1396	1477
nuclei	188	619	328	874
cell total	960	1680	1724	1351
% uptake	5.59	10.25	11.30	9.91

SDS polyacrylamide gel electrophoresis was also employed to demonstrate the presence of full length peptide in cells 6 days after exposure of cells to labelled carrier peptide. This represents greatly enhanced stability in biological systems compared to native peptides composed of L-amino acids.

Example 3 - Distribution of peptide in mammalian tissues

Distribution of the C-14 labelled peptide (example 2) in mice was determined by injecting mice with 0.1 uCi of labelled carrier peptide intravenously (IV) (see Figure 1) and subcutaneously (SC) (see Figure 2). Mice were sacrificed at specific times following injection over a 48 hou. period. The major internal organs were removed, weighed and solubilized using a tissue solubilizer designed to be compatible with scintillation cocktail. The amount of peptide in each sample was determined using scintillation counting. Results are graphed in Figures 1 and 2.

These studies demonstrated rapid and efficient distribution of the carrier peptide to all major organs of the injected mice. The primary sites of deposition of carrier peptide being the liver, kidneys, spleen and lungs. Accordingly, the carrier peptide can most usefully be employed as a carrier for a biochemical agent intended for delivery in these tissues.

WE CLAIM:

1. A chemical conjugate, comprising at least one biological agent to be delivered to an intracellular environment, and a carrier peptide coupled chemically therewith to facilitate said delivery, wherein said carrier peptide is comprised principally of positively charged amino acids, at least 50% of which are in the D-isomer form.

2. A chemical conjugate according to claim 1, wherein said carrier peptide consists of 8, 9 or 10 D-arginine residues.

3. A chemical conjugate according to claim 1, wherein said carrier peptide consists of nine D-arginine residues.

4. A chemical conjugate according to claim 1, wherein said biological agent is a polypeptide.

5. A chemical conjugate according to claim 2, wherein said biological agent is a polypeptide.

6. A chemical conjugate according to claim 3, wherein said biological agent is a polypeptide.

7. A chemical conjugate according to claim 1, wherein said biological agent is a polynucleotide.

8. A chemical conjugate according to claim 2, wherein said biological agent is a polynucleotide.

9. A chemical conjugate according to claim 3, wherein said biological agent is a polynucleotide.

10. A chemical conjugate according to claim 1, comprising a single biochemical agent.

11. A chemical conjugate according to claim 10, wherein said biochemical agent is coupled to the N-terminus of said carrier peptide.

12. A pharmaceutical composition comprising chemical conjugate as defined in claim 1 combined with a pharmaceutically acceptable excipient.

FIGURE 1. Pharmacokinetics of C-14 Peptide in IV-Injected Mice

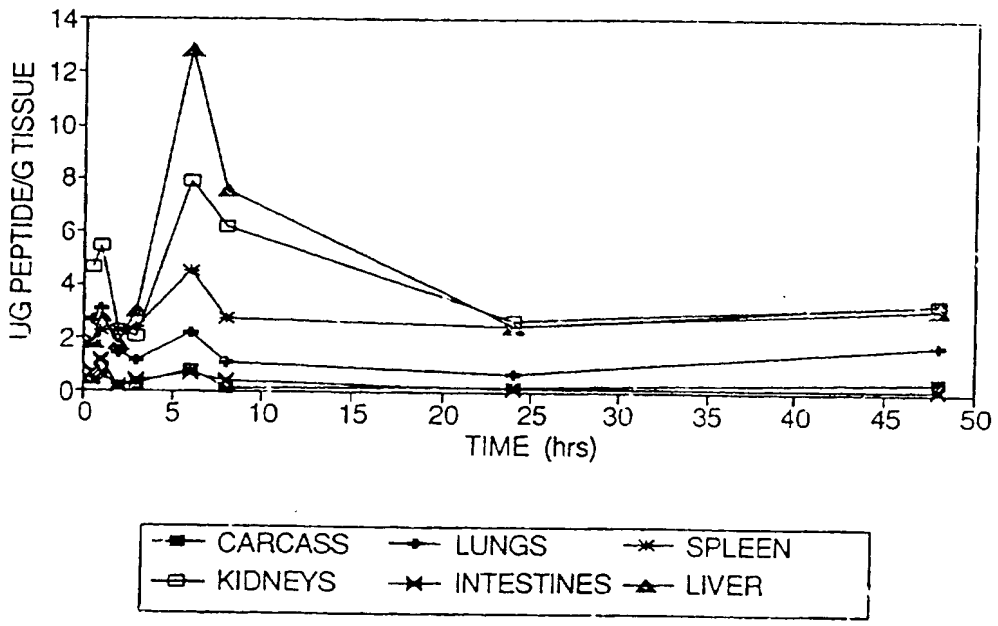

FIGURE 2. Pharmacokinetics of 14-C Peptide in SubQ-Injected Mice